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Mating triggers dynamic immune regulations in wood ant queens

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sexual conflict;
sperm survival.

Abstract

Mating can affect female immunity in multiple ways. On the one hand, the immune system may be activated by pathogens transmitted during mating, sperm and seminal proteins, or wounds inflicted by males. On the other hand, immune defences may also be down-regulated to reallocate resources to reproduction. Ants are interesting models to study post-mating immune regulation because queens mate early in life, store sperm for many years, and use it until their death many years later, while males typically die after mating. This long-term commitment between queens and their mates limits the opportunity for sexual conflict but raises the new constraint of long-term sperm survival. In this study, we examine experimentally the effect of mating on immunity in wood ant queens. Specifically, we compared the phenoloxidase and antibacterial activities of mated and virgin *Formica paralugubris* queens. Queens had reduced levels of active phenoloxidase after mating, but elevated antibacterial activity 7 days after mating. These results indicate that the process of mating, dealation and ovary activation triggers dynamic patterns of immune regulation in ant queens that probably reflect functional responses to mating and pathogen exposure that are independent of sexual conflict.

Introduction

Mating and immunity are central for fitness, and interestingly the two processes influence each other in multiple ways (Lawniczak *et al.*, 2007). Research in the field of sexual selection has long suggested that individuals choose partners on the basis of traits signalling their heritable immunity (Hamilton & Zuk, 1982; Folstad & Karter, 1992; Møller *et al.*, 1999; Blount *et al.*, 2003; Koskimaki *et al.*, 2004; Rantala & Kortet, 2004). Conversely, recent empirical results indicate that mating influences immunity, either directly or through various physiological modifications, constraints and trade-offs (Rolff & Siva-Jothy, 2002; Lawniczak *et al.*, 2007).

Mating has been shown to either induce or suppress distinct components of the female immune system in various invertebrate species (reviewed in Fedorka *et al.*, 2004; McGraw *et al.*, 2004; Fedorka *et al.*, 2007; Lawniczak *et al.*, 2007). The patterns of post-mating immune regulation vary greatly across taxa, and so far the proximate mechanisms and adaptive value of these modulations remain poorly understood, which calls for further studies in systems that differ in mating systems and ecology (Lawniczak *et al.*, 2007).

After mating, females may have to down-regulate their investment in immune defences in order to allocate more resources to reproduction or cope with other physiological constraints (Sheldon & Verhulst, 1996; Rolff & Siva-Jothy, 2002). A negative correlation between reproduction and immunity has been found in a variety of vertebrate (Norris & Evans, 2000; French *et al.*, 2007) and insect species (Siva-Jothy *et al.*, 1998; Fedorka *et al.*, 2004; Calleri *et al.*, 2007). This immune suppression has been suggested to be one of the indirect costs of reproduction as it can decrease survival and future reproduction (Sheldon & Verhulst, 1996; Rolff & Siva-Jothy, 2002). In contrast, the female's immune system may also be up-regulated after mating in order to fight pathogens transmitted during mating (Altizer *et al.*, 2003; Knell & Webberley, 2004) or heal copulatory wounds (Crudgington & Siva-Jothy, 2000; Reinhardt & Siva-Jothy, 2007).

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A further major factor that can influence the relationship between mating and immunity is sexual conflict (Chapman, 2006; Lawniczak *et al.*, 2007). In various mammals and insect species, males transfer active substances with their ejaculate in order to increase female investment in reproduction or influence sperm competition, sometimes at the expense of the female longevity or immunity (Chapman *et al.*, 1995; Heifetz *et al.*, 2000; Chapman, 2001; Rolff & Siva-Jothy, 2002; Poiani, 2006). In *Drosophila* for instance, some seminal proteins are toxic and others interfere with the female's immunity in complex ways, up-regulating some immune genes while down-regulating others (Lung *et al.*, 2002; McGraw *et al.*, 2004; Peng *et al.*, 2005; Mueller *et al.*, 2007). Sperm may be recognized as nonself by the female immune system (Naz *et al.*, 1993; Poiani, 2002; McGraw *et al.*, 2004), and males may transfer immune suppressants with their ejaculates in order to increase their sperm survival (Barratt & Pockley, 1998; Fedorka & Zuk, 2005). Furthermore, in polyandrous species the immune reaction towards sperm may allow cryptic female choice of the sperm that best withstand the immunological attack (Birkhead & Pizzari, 2002; Poiani, 2002).

Social Hymenoptera, and particularly ants, are interesting models to study the interactions between mating and immunity, because of their very peculiar reproductive biology. Typically, ant queens mate at the beginning of their adult life and males die shortly after mating. Queens store sperm in a special organ, the spermatheca, and use it to fertilize thousands or even millions of offspring over their entire reproductive lifespan, which can span from a couple to as many as 29 years (Hölldobler & Wilson, 1990; Keller & Genoud, 1997).

As ant queens never, or at most very rarely, remate, there is a life-long commitment between queens and their mates, so that when the female dies or loses her fertility, the male reproductive success comes to an end as well (Boomsma *et al.*, 2005). Such long term and exclusive partner commitment strongly reduces the potential for sexual conflict, as ant males would not benefit from manipulating queens into increasing their short-term reproduction to the detriment of their longevity (Boomsma *et al.*, 2005). Mating indeed increased the longevity of queens in the ant *Cardiocondyla obscursior*, even if this species has unusually long-lived males that can remate (Schrempf *et al.*, 2005).

Ant queens do face two other types of somewhat opposing challenges. First, they have to store millions of spermatozoa for several years. The cost of sperm storage may result in a decreased investment in immunity, and indeed the quantity of sperm stored was negatively correlated with the ability to encapsulate a nylon filament in the leaf-cutting ant *Atta colombica* (Baer *et al.*, 2006). Ant queens may also down-regulate some specific immune components that are detrimental to sperm survival, while up-regulating protective mechanisms. For example, antioxidative enzymes are over-expressed in the spermatheca of honeybee queens (Collins *et al.*, 2004). Second, ant queens probably face an elevated risk of parasitism during mating and colony foundation, which should select for high levels of immune defences. Leaf-cutting ant queens indeed increase their encapsulation response during the early stage of colony founding, probably to defend themselves against soil opportunistic pathogens (Baer *et al.*, 2006).

To sort among the diversity of potential processes linking mating and immunity, it is important to obtain new empirical data on post-mating immune regulation in species with particular mating systems and ecology. The wood ant *Formica paralugubris* is unicolonial, with many queens per nest (Chapuisat *et al.*, 1997; Holzer *et al.*, 2006). New queens and males are produced once per year, in late spring and early summer. Queens and males have alternative dispersal strategies. They mate either within the natal nest or on meadows that they reach on the wing (Cherix *et al.*, 1991; Chapuisat, 1998; Chapuisat & Keller, 1999). In both situations the majority of queens mate with a single male, with occasional cases of double and triple mating, corresponding to an effective mating frequency of 1.13 (Chapuisat, 1998). After mating, queens stay in their natal nests or seek adoption in other nests of *F. paralugubris* or *Serviformica* species (Cherix *et al.*, 1991; Chapuisat, 1998; Chapuisat & Keller, 1999). They shed their wings and start to lay eggs shortly after mating (Cherix *et al.*, 2006). Later on, queens and workers can leave on foot to establish new nests by budding (colony budding, Bourke & Franks, 1995; Chapuisat *et al.*, 1997). Overall, the biology of *F. paralugubris* should be associated with reduced level of sexual conflict, moderate parasite exposure during mating, moderate energetic constraints because queens do not found colonies independently, and mechanisms to ensure sperm survival over several years. Hence, *F. paralugubris* queens face relatively mild constraints compared with species such as *Atta colombica,* which mate with many males and found colonies independently (Baer *et al.*, 2006).

In this study, we investigate how mating affects the immune response of *F. paralugubris* ant queens. We compared the immune defences of queens that were experimentally allowed to mate and of queens that had to remain virgin. We monitored how two components of the immune response varied over time: phenoloxidase, a constitutive defence, and antibacterial activity, an inducible response. Phenoloxidase is involved in wound healing and might thus be activated before or after mating, whereas the production of antibacterial peptide will depend on exposure to bacteria. These results will shed light on the dynamics of post-mating immune modulation in a species with reduced level of sexual conflict.

Methods

Sampling and controlled mating of queens

We sampled *F. paralugubris* queens and males from nests in the Jura mountains in Switzerland. Queens came from five nests near the Chalet à Roch (N46°32′32″, E06°11′08″) and from two nests in the Bois de Peney (see Holzer *et al.*, 2006). Males were collected from two nests in each location. All queens and males were sampled in June 2006, in the early morning, upon emergence on the surface of the nests. Queens sampled on the nest surface could have either engaged in a nuptial flight or returned inside the nest, especially in case of weather change.

The queens from each nest were randomly allocated to either the *mated* or *virgin* treatment. In the *mated* treatment, nestmate queens were placed with an equal number of males originating from another nest of the same location in a plastic container (21 × 37 × 18 cm) covered with a thin net. In total, 133 queens from seven nests were allowed to mate, in seven groups. In the *virgin* treatment, nestmate queens were placed in an identical container, but without males. In total, 125 queens from the same seven nests were subjected to the *virgin* treatment. In both treatments, the containers were placed for 40 min under direct sunlight, which stimulates mating (Cherix *et al.*, 2006). Queens from each nest and treatment were then kept in separate plastic boxes (13.5 × 15 × 5 cm) and brought back to the laboratory. They were kept at 25 °C, with *ad libitum* water and under a 12 h day/night cycle, until haemolymph extraction. Queens had no access to food and had to rely on their energetic reserves, which should permit to observe trade-offs in immunity that might be undetectable when abundant resources allow for resource compensation.

We extracted haemolymph from subsamples of queens 2 h, 36 h and 7 days after the treatments. After haemolymph extraction, the queens were dissected to check for their mating status. The spermatheca of queens is transparent when virgin and opaque after mating (Gösswald, 1989). Overall, 99.2% (124 of 125) of the queens subjected to the *virgin* treatment were effectively virgin and 85.7% (114 of 133) of the queens subjected to the *mated* treatment had mated. The mated queen from the *virgin* treatment and the virgin queens from the *mated* treatment were removed from the analyses. When sampled for haemolymph extraction, most of the virgin queens (96.8%; 120 of 124) still had their wings whereas most of the mated queens (82.5%; 94 of 114) were wingless. There was no significant difference in queen mortality between treatments. Specifically, 4.3% and 5.3% of the queens subjected to the *mated* and *virgin* treatment died during the course of the experiment, respectively ($\chi^2_1 = 0.14$, $P = 0.71$).

Immune measures

One microliter of haemolymph was collected by puncturing ice-cooled queens between the third and fourth tergite with a heat-sharpened glass capillary. Haemolymph was immediately flushed into a 0.5 mL Eppendorf tube containing 10 μL of ice-cold Sodium Cacodylate buffer (Na-Cac: 0.01 M; $CaCl_2$: 0.005 M) that was snap-frozen in liquid nitrogen and stored at −80 °C.

Phenoloxidase (PO) activity was measured from the haemolymph sampled 2 h, 36 h and 7 days after the mating vs. virgin treatments. PO is present in two forms in insect haemolymph: an active form (active PO), which catalyzes enzymatic reactions, and an inactive form, prophenoloxidase (proPO) which is activated as a response to parasite entry (Soderhall & Cerenius, 1998). Active PO is measured as the increase in optical density induced by the transformation of L-DOPA into dopachrome by phenoloxidase present in the sample. Total PO (i.e. active PO + proPO) is measured in the same way but after activation of the inactive form of the enzyme by trypsin.

Active PO and total PO were measured with a spectrophotometer for 96-well microplates (iEMS reader MF, Labsystems, Helsinki, Finland). To measure active PO, 3 μL of haemolymph solution were mixed with 80 μL of water and 10 μL of phosphate-buffered saline in a microplate well. For the total PO, trypsin was added to water (0.25%) and the mix was left for 5 min at room temperature. Ten microlitres of L-DOPA (4 mg mL^{-1}) were added to the mix. The absorbance at 492 nm was measured every 10 s for 50 min at 30 °C. The active PO and total PO were measured as the slope of the reaction curve during the linear phase of the reaction, i.e. the rate of change of optical density per second (Moret & Siva-Jothy, 2003).

The antibacterial activity of haemolymph sampled 36 h and 7 days after treatment was measured with an inhibition zone assay (Moret & Schmid-Hempel, 2000). This assay consists in depositing two microliters of haemolymph solution on a culture medium inseminated with live bacteria (10 g bactotryptone, 10 g bacto-agar, 10 g NaCl, 5 g yeast extract, 1000 mL distilled water, pH 7.5; mixed with the bacteria *Arthrobacter globiformis* and adjusted to 10^5 cells mL^{-1}). The antibacterial activity of the sample is measured after overnight incubation at 30 °C as the surface of the zone where bacterial growth has been inhibited.

Statistical analysis

Total PO and active PO were analysed with two-way ANOVA mixed models. Mating status (mated or virgin) and time (2 h, 36 h or 7 days) were used as fixed factors, whereas box (i.e. the plastic box in which queens from a single nest and from the same treatment group were kept) was used as random factor. Data were log-transformed to fulfil the normality assumption. Within

each time category, the difference between mated and virgin queens was tested with *post-hoc* Tukey tests. Differences in antibacterial activity of mated and virgin queens were tested with Wilcoxon rank sum tests, because the data were not normally distributed and had heterogeneous variances. All statistical analyses were performed with JMP 6.0 (SAS Institute Inc., Cary, NC, USA).

Results

Phenoloxidase

Overall, the level of total PO was not significantly different between mated and virgin queens, even if there was a trend for lower total PO in mated queens (Mating status effect: Table 1a; Fig. 1a). Mated and virgin queens did not differ significantly in their level of total PO within any of the three sampling times (*post-hoc* Tukey tests: all $P > 0.05$).

Globally, mated queens had significantly lower levels of active PO than virgin ones (Mating status effect: Table 1b, Fig. 1b). However, mated and virgin queens did not differ significantly in their level of active PO within any of the three sampling times (*post-hoc* Tukey tests: all $P > 0.05$). The significant interaction between mating status and time (Table 1b) indicates that active PO of mated and virgin queens had different temporal dynamics. Specifically, virgin queens had a high initial level of active PO, which decreased significantly over time (ANOVA mixed model within virgin queens: effect of time, $F_{2,\ 114.8} = 6.92$, $P = 0.001$; Fig. 1b). In contrast, mated queens had a low level of active PO, which was stable over time (ANOVA mixed model within mated queens: effect of time, $F_{2,\ 104.8} = 0.67$, $P = 0.51$; Fig. 1b).

Antibacterial activity

Overall, mated queens had a higher antibacterial activity than virgin ones (Wilcoxon rank sum test: $N_{\text{virgin}} = 76$, $N_{\text{mated}} = 79$, $Z = -6.42$, $P < 0.0001$, Fig. 2). This difference was due to the much higher antibacterial activity of mated queens 7 days after mating ($N_{\text{virgin}} = 60$, $N_{\text{mated}} = 53$, $Z = 7.18$, $P < 0.0001$, Fig. 2). Mated queens indeed showed a significant increase in their level of antibacterial activity over time, whereas the antibacterial activity of virgin queens remained low (Mated queens:

Table 1 Effect of mating status and time on phenoloxidase (PO).

	d.f. num	d.f. den	*F*	*P*
(a) Total PO				
Mating status	1	44.61	3.71	0.061
Time	2	230.2	4.10	0.018
Mating status * Time	2	230.2	1.40	0.250
(b) Active PO				
Mating status	1	58.9	4.41	0.040
Time	2	220.4	2.25	0.108
Mating status * Time	2	220.4	6.10	0.003

Two-way mixed models for (a) total PO and (b) active PO.

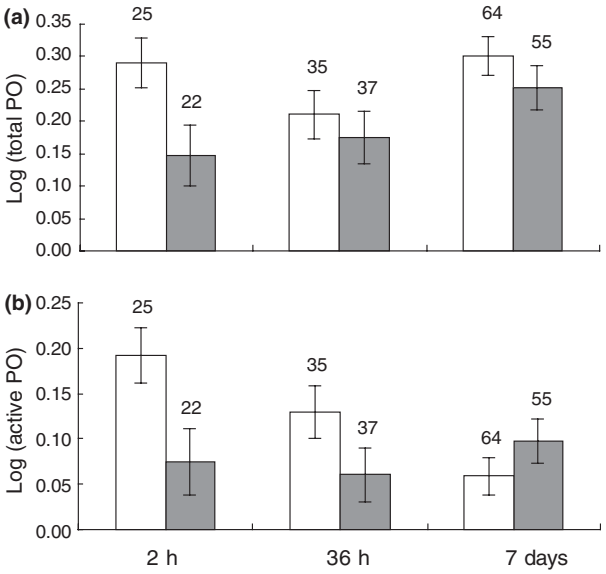

Fig. 1 Total PO ± SE (a) and active PO ± SE (b) for virgin queens (white bars) and mated queens (grey bars) 2 h, 36 h and 7 days after mating. Enzyme activity is expressed as the rate of change of optical density $\times 10^2$ per second. Sample sizes (number of queens) are indicated above the bars.

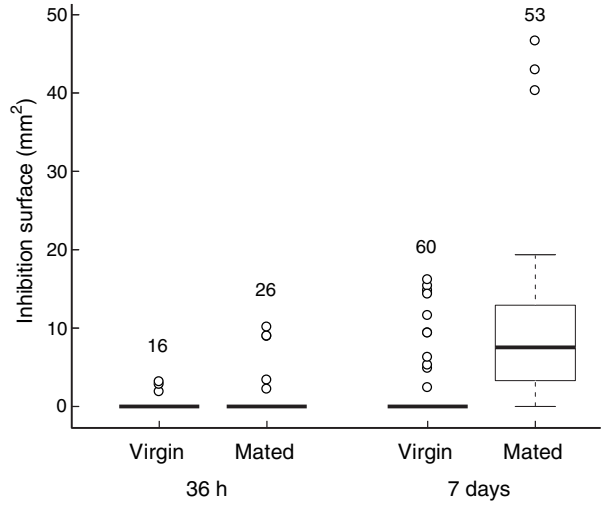

Fig. 2 Antibacterial activity expressed as the surface of inhibition of virgin and mated queens, 36 h and 7 days after experimental mating flight. Boxes correspond to the interquartile range and solid lines to the median for each group. Whiskers contain all points that are not further from the box than 1.5 times the interquartile range. Sample sizes (number of queens) are indicated above the bars.

$N_{36h} = 26$, $N_{7d} = 53$, $Z = -4.99$, $P < 0.0001$; Virgin queens: $N_{36h} = 16$, $N_{7d} = 60$, $Z = 0.16$, $P = 0.88$, Fig. 2).

Discussion

Mating is associated with drastic behavioural and physiological changes in ant queens (Hölldobler & Wilson, 1990). Virgin queens usually fly away from the confined environment of their natal colony, mate to acquire a lifetime supply of sperm and then either found a new colony or try to be accepted in an already established one. Our study shows that the process of mating, which includes exposure to males, copulation, ejaculate transfer, wing shedding and ovaries activation, has a major impact on the immune defences of wood ant queens.

One or more of these steps in the mating process triggers a multifaceted pattern of immune regulation in wood ant queens, with a low level of active phenoloxidase after mating and an increase in antibacterial activity 7 days after mating. These changes in opposite directions for two components of the immune defenses are unlikely to be due to sexual conflict and most likely reflect functional responses to the challenge of mating.

Several nonmutually exclusive hypotheses might explain the low level of active PO after mating. First, this may come from a physiological antagonism between immunity and egg production mediated through juvenile hormone, as has been documented in the mealworm beetle *Tenebrio molitor* (Rolff & Siva-Jothy, 2002). Second, the PO enzyme might have been used to melanize wounds inflicted on the female genital tract during copulation (Moreau *et al.*, 2000; Plaistow *et al.*, 2003; Theopold *et al.*, 2004), which is consistent with the fact that the genitalia of mated *Formica* queens show melanized lesions (Kamimura, 2008), and that the prophenoloxidase cascade is a constitutive insect immune defence which is quickly available for wound healing and parasite sequestration (Cerenius & Soderhall, 2004; Lemaitre & Hoffmann, 2007). A third, and probably less likely explanation is that the queens down-regulate the level of active PO after mating in order to increase sperm survival.

Virgin queens had a high level of active PO two hours after the treatment. This is somewhat surprising because phenoloxidase is generally stored in its inactive form (proPO), which is only activated upon request (Soderhall & Cerenius, 1998). This result suggests that an important portion of the proPO is turned into active PO in young virgin queens before mating, and that this PO will then be used to heal copulatory wounds or cope with other challenges associated with mating, dispersing and starting up reproduction.

Queens of *F. paralugubris* did not increase their level of active PO 7 days after mating, which contrasts with the higher encapsulation rate of *A. colombica* queens 9 days after mating (Baer *et al.*, 2006). This difference might be due to the distinct mode of colony founding of the two species, as *A. colombica* queens are exposed to soil pathogens during independent colony founding, whereas *F. paralugubris* queens seek adoption into existing nests and were kept in pathogen-free boxes after mating in our experimental setting.

Mated queens had a high antibacterial activity 7 days after mating, in contrast to virgin queens, which kept a very low level of antibacterial activity. The antibacterial activity of the haemolymph is due to the synthesis of antibacterial peptides in response to bacterial or fungal infections (Gillespie *et al.*, 1997). It seems however unlikely that the high antibacterial activity of mated queens was induced by the transmission of bacteria during copulation, because the synthesis of antibacterial peptides occurs rapidly after an immune challenge, and the antibacterial activity was still low 36 h after mating (Vilcinskas *et al.*, 1999; Korner & Schmid-Hempel, 2004; Lavine *et al.*, 2005). This delayed up-regulation of the antibacterial activity is consistent with an infection occurring during the days following mating, possibly through copulatory wounds (Kamimura, 2008), or with an adaptive, preventive response to future infection risks. It might also be due to the large-scale breakdown of tissues and cells associated with wing shedding and wing muscles histolysis, which releases intracellular bacteria, free radicals or other components that may activate the immune system (Jones *et al.*, 1978; Tian *et al.*, 2004).

Independently of the mechanisms underlying this induction, the high antibacterial activity, which may last for several days (Boman & Hultmark, 1987), is likely to protect recently mated queens against pathogen exposure when they disperse and seek adoption in established colonies. In line with this idea, Tian *et al.* (2004) suggested that the up-regulation of two antimicrobial peptide genes after mating may protect fire ant queens against infection.

Evidence from other insect species suggest that the induction of some antibacterial peptides following mating may be a general process (Lawniczak *et al.*, 2007). In *Drosophila*, several gene expression studies have shown that copulation, as well as sperm and accessory gland products, induce an up-regulation of antibacterial genes (Lawniczak & Begun, 2004; McGraw *et al.*, 2004; Peng *et al.*, 2005; Fedorka *et al.*, 2007). These results have been discussed in the context of sexual conflict and male manipulation (McGraw *et al.*, 2004; Fedorka *et al.*, 2007). Our finding that a similar post-mating induction of antibacterial peptides occurs in a species with minimal level of sexual conflict indicates that this process may also be a functional response to an actual infection or to an elevated infection risk (Tian *et al.*, 2004; Peng *et al.*, 2005). This suggests that the level and effect of infections should be assessed before concluding that males manipulate female immune systems in order to maximize their own fitness.

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